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ORIGINAL ARTICLE

Immunological characterization of a rigid α-Tn mimetic on murine iNKT and human NK cells

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Abstract The ability of a rigid α-Tn mimetic (compound **1**) to activate murine invariant natural killer T (iNKT) and human natural killer (NK) cells, two subsets of lymphocytes involved in cancer immunesurveillance, was investigated. For this purpose, the mimetic **1** was properly conjugated to a stearic acid containing glycerol-based phospholipid (compound **5**) to be presented, in the context of the conserved non polymorphic major histocompatibility complex class I-like molecules (CD1d), to iNKT cells. On the contrary, the mimetic **1** was conjugated to a multivalent peptide-based scaffold (compound **6)** to induce NK cell activation.

Keywords Invariant natural killer T cells · Natural killer cells · α-Tn antigen · Glycomimetic · Immunotherapy

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Introduction

During the last decade, substantial pieces of evidences showed the importance of the immune system in fighting cancer. The immune system can, indeed, regulate tumor development/suppression through a sequence of complex events that starts with the recognition of tumor cells and goes on with a crosstalk among immune cells. This process may lead to immune cell infiltration within the tumor with possible malignant cell killing or conduct to a temporary state of dynamic equilibrium between immunosurveillance and tumor growth. If the immune system fails to eliminate tumors completely, tumors try to protect themselves by creating a permissive microenvironment and a resistant tumor phenotype able to escape the immunosurveillance [1, 2]. In this framework, the immunotherapy, an old therapeutic approach [3] improved in the last few years, appeared to be an appealing strategy [4] to circumvent the tumor-induced immune suppression through the breakthrough of the "checkpoint blockades" [5–7].

A multi-targeting approach to fight tumor cells is, at present, of growing interest [8–13]. For example, the delivery of tumor-targeted antigens [11, 13] through the introduction of targeting moieties covalently linked to tumor antigens. This procedure could drive antigen-specific immune responses by harnessing stimulatory functions of specific subsets of immune cells. Synthetic architectures can be assembled to bind to specific receptors on immune cells, or spatial arrangement/multivalent presentations of conjugated antigens can be designed.

Among other cells of the immune system, invariant natural killer T (iNKT) and natural killer (NK) cells, consisting of a subset of lymphocytes, play essential roles in immunesurveillance and represent potential targets for

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immunotherapy. These cells, even if they share some phenotypical and functional similarities, exert non-redundant and specific functions [14].

In particular, human iNKT cells are a small proportion of T lymphocytes, expressing αβ T cell receptor (TCR) and natural killer (NK) cell lineage markers (*e.g.*, CD161), which exhibit functional properties of both conventional T and NK cells [15]. They express an invariant TCR-α chain (Vα24-Jα18), paired with the semi-invariant TCR-β chain (Vβ11), that recognize lipid antigens when presented in the context of the conserved non polymorphic major histocompatibility complex class I-like molecules (CD1d) [16, 17]. Despite their invariant TCR, iNKT cells are able to recognize a diverse range of antigens, ranging from foreign microbials to endogenous lipids [18], and rapidly and specifically respond against stimulation. Depending on the structure of lipid antigens and/or cytokine stimuli iNKT cells can release a diverse range of cytokines (*i.e.*, IFN-γ, IL-4) and chemokines (*i.e.*, RANTES, eotaxin) [19], and up-regulate different costimulatory molecules (*i.e.*, CD28, CD40L) [20], as well as cytotoxicity receptors (*i.e.*, NKG2d) and mediators (*i.e.*, perforine and granzyme) [21]. Through these signals iNKT cells can influence the activation/differentiation of dendritic cells (DCs), B and T lymphocytes, as well as NK cells, macrophages, and granulocytes. In the context of tumor immunity, iNKT cells play a critical role in immunosurveillance, even if the mechanisms underlying iNKT cell activation during tumor growth remain elusive.

On the other hand, NK cells are lytic lymphocytes, which mediate rapid effector responses without any priming [22, 23]. NK cells, compared to T or B lymphocytes, do not express clonally distributed receptors for antigens, [24, 25] but they have a plethora of germ-line encoded inhibitory/activating receptors [26]. In particular, the inhibitory receptors recognize the self-major histocompatibility complex class I (MHC-I) molecules preventing NK cell activation, while the activating receptors recognize the pathological cells, having the MHC-I molecules down-regulated [27] and/or the "stress-induced" self proteins up-regulated [28]. NK cells are able to kill dangerous cells in few minutes by two main mechanisms: i) the interaction of specific cell surface ligands (*e.g.*, FasL) with death receptors (*e.g.*, Fas/CD95) expressed on transformed/damaged cells; ii) the release of lytic granules, containing perforin and granzyme molecules, which can destroy target cells within hours trough cytotoxic activities similar to that of CD8+ cytotoxic T lymphocytes [29]. In addition, NK cells, once activated, release various cytokines/chemokines able to influence the functions of both adaptive and innate immune system [30–33]. Recently, the immunological role of these cells appeared to be more complex, extending beyond the classical "innate" time-frame towards functional features of adaptive immunity, such as longevity and immunological memory [34–36].

On the basis of these evidences, the aim of the present study was to investigate the activity of the fully synthetic antigen **1** [37, 38] (Fig. 1) on iNKT and NK cell activation. Compound **1** is the first example of a conformationally constrained mimetic of the well-studied α-Tn-antigen **2** [39], a hapten expressed on different kind of cancer cells.

Recently, some of us successfully demonstrated that the Tn mimetic **1** (Fig. 1), when properly functionalized on the carboxylic group with a suitable architecture, is able to trigger different immune cells and induce specific and long-lasting immune responses [40]. In particular: i) biocompatible and immunogenic iron oxide superparamagnetic nanoparticles **3** (Fig. 1), decorated with high density of the Tn mimetic **1**, induce a specific macrophage activation [41]; ii) the synthetic peptide-based glyco-cluster **4** (Fig. 1), which provided the multivalent presentation of the mimetic **1**, elicits significant IgG/IgM mAb production and protection in mice affected by breast cancer [40]. The overall data confirm that the biological effect of the Tn-mimetic **1** can be modulated by changing the nonglycan portion of the conjugate.

Thus, following this concept and looking for an adequate and multi-target immune response, we here investigated the ability of the rigidified mimetic **1** to induce iNKT and NK cells activation. To gain this goal, both the glycoconjugates **5** and **6** were selected (Fig. 2). In particular, the glycolipid **5,** which consists of a stearic acid containing glycerol-based phospholipid conjugated to a residue of the Tn-mimetic **1**, was chosen because of its ability to be presented by CD1d [42] to induce iNKT cell activation (T-cell-antigen presenting assay). On the contrary, taking into account that the multivalent presentation of **1** is important to modulate the induced immune response [40] the hexavalent glycopeptide 6 was also included in the study. Indeed, the glycopeptide **6** consists, of a cyclopeptide scaffold, termed regioselectively addressable functionalized template (RAFT) [43], where six residues of **1**, four on the upper face and two on the lower face of the RAFT, were conjugated to the lysine lateral chains. The ability of **6** to activate NK cells was assessed by measuring the expression of CD107a (degranulation assay).

Materials and methods

Materials

Reagents were purchased from commercial suppliers and used without purification. ESI-MS mass spectra were recorded on a LCQ-Fleet Ion Trap equipped with a standard Ionspray interface from Thermo Scientific. NMR spectra were recorded on Varian Gemini 300,

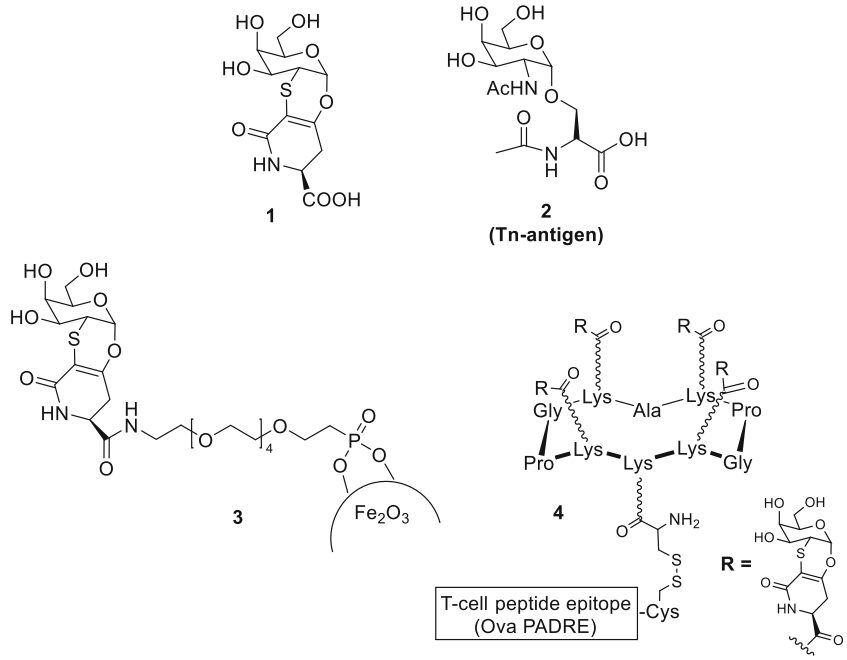


Fig. 1 Structure of the Tn-mimetic **1**, the native Tn antigen **2**, the iron oxide superparamagnetic nanoparticles **3**, and glycopeptide **4**

Mercury Plus 400, Bruker AVANCE 500. [α]D values were measured using a JASCO DIP-370 instrument. Protected amino acids and Fmoc-Gly-Sasrin resin were obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). PyBOP was purchased from France Biochem. Reaction progress was monitored by reverse-phase HPLC on Waters equipment using C_{18} columns. Analytical and preparative separation was carried out at 1.0 mL/min (EC 125/3 nucleosil 300–5 C_{18}) and at 22 mL/min (VP 250/21 nucleosil 300–7 C_{18}) with UV monitoring at 214 nm and 250 nm using a linear A–B gradient (buffer A: 0.09% CF_3CO_2H in water; buffer B: 0.09% CF_3CO_2H in 90% acetonitrile). Cyclopeptides were analyzed by mass spectrometry using electrospray ionization on an Esquire 3000+ Bruker Daltonics in positive mode.

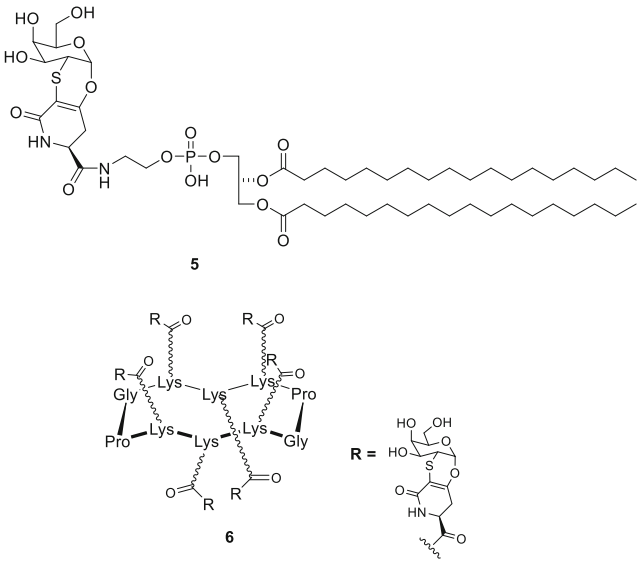


Fig. 2 Structure of the glycolipid **5** and the glycopeptide **6**

Chemistry

Synthesis of **9**

To a stirred solution of TBTU (0.033 g, 0.104 mmol) in dry DMF (0.6 mL) NMM (0.012 mL, 0.104 mmol) was added. The mixture was left strirring for 15 min at r.t than a solution of **7** (0.024 g, 0.052 mmol) in DMF (0.4 mL) was added. The reaction mixture was left stirring for 10 min than a solution of **8** in dry CH_2Cl_2:$CHCl_3$ (3:1) was added. The mixture was warmed at 40 °C for 1 h then cooled to r.t and left stirred for 15 h. After this time the reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with H_2O (1 × 10 mL), a 1 M solution of Triethylammonium bicarbonate (1 × 15 mL). The organic phase was dried over Na_2SO_4 and concentrated to dryness to give a crude which was purified by flash chromatography on silica gel (AcOEt +0.1% NEt_3 ➔ DCM: MeOH 5: 1 + 0.1% NEt_3). Cationc exchange of the triethylammonium salt of **9** with amberlite resin IR-120 Na^+ ($CHCl_3$: MeOH 2:1) gave **9** (0.028 g, 0.023 mmol, 45%) as a glassy solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.46 (bs, 1H), 5.74 (as, 1H, H-1), 5.39 (as, 1H, H-4), 5.28 (bs, 1H, H-2‴), 5.02 (ad, J = 10.8 Hz, 1H, H-3), 4.39–4.46 (m, 1H, H-5), 4,39–4.27 (m, 1H, H-3‴a), 4.17–4.11 (m, 4H, H-6a, H-6b, H-3‴b, H-2′), 3.90 (bs, 4H, H-1″,

H-1‴), 3.57 (d, J = 11.7 Hz, 1H, H-2), 3.49–3.41 (m, 2H, H-2″), 3.11–3.04 (m, 3H, H-3'a, CH_2CO), 2.93 (bs, 1H, H-3'b), 2.33–2.27 (m, 6H, CH_2), 2.16 (s, 3H, COOCH_3), 2.07 (s, 3H, COOCH_3), 2.03 (s, 3H, COOCH_3), 1.59 (bs, 4H, CH_2), 1.27 (bs, 54H, CH_2), 0.89 (t, J = 7 Hz, 6H, CH_3): ^{13}C NMR (125 MHz, $CDCl_3$) δ: 173.5 (Cq), 170.6 (Cq), 170.3 (Cq), 169.9 (Cq), 169.8 (Cq), 165.4 (Cq), 95.4 (C-1), 70.58 (C-2‴), 68.7 (C-5), 67.1 (C-4), 66.1 (C-3), 64.0 (C-2″,C-1‴), 62.61 (C-3‴,C-6), 61.45 (C-1‴), 51.82 (C-2′), 45.69 (*C*H2), 36.22 (C-2), 34.35, 34.0, 31.9, 29.7, 29.6, 29.6, 29.6, 29.4, 29.3, 29.3, 29.2, 29.2, 24.9, 24.8, 22.6, 20.6, 20.5, 18.4, 14.0, 8.5, 0.9: ^{1}P NMR (500 MHz, $CDCl_3$) δ: 1.42 (s): ESI-MS: m/z Calcd for $C_{59}H_{101}N_2O_{18}PS$: 1186.64 $[M - H]^-$; found: 1187.36.

Synthesis of **5**

To a stirred solution of **9** (23.0 mg, 0.019 mmol) in a mixture MeOH:$CHCl_3$ (5:1, 1.2 mL) 0.953 mL of a K_2CO_3 solution (0.02 M in MeOH) were added. The reaction mixture was stirred for 1 h at rt. then a 0.01 M solution of acetic acid in MeOH was added to reach the pH = 7. The crude was purified by gel filtration on Sephadex LH-20 column (2 × 20, d x h) (MeOH) to give **5** (9.1 mg, 0.0085 mmol, 45%) as white glassy solid. $[\alpha]^{25}_D$ = −21.4 (c 0.15 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.69 (d, $J_{1,2}$ = 2.7 Hz, 1H, H-1), 5.24–519 (m, 1H, H-2‴), 4.43–4.40 (A part of an ABX system, $J_{A,B}$ = 12.1 Hz, $J_{A,X}$ = 3.15 Hz, 1H, H-3‴'a), 4.20–4.16 (m, 2H, H-2′, H-3‴'b), 4.08–3.96 (m, 4H, H-4, H-5, H-2″ or H-1‴), 3.83–3.79 (A part of an ABX system, $J_{A,B}$ = 11.5 Hz, $J_{A,X}$ = 6.0 Hz, 1H, H-6a), 3.79–3.76 (B part of an ABX system, $J_{B,A}$ = 11.5 Hz, $J_{B,X}$ = 6.0 Hz, 1H, H-6b), 3.66–3.62 (m, 3H, H-3, H-2″ or H-1‴), 3.42–3.38 (m, 1H, H-1"a), 3.47 (dd, $J_{2,1}$ = 10.7 Hz, $J_{2,3}$ = 2.8 Hz, 1H, H-2), 3.53–3-50 (m, 1H, H-1"b), 2.86 (ad, J = 7.0 Hz, 2H, H-3'a, H-3'b), 2.33–26 (m, 2H, CH_2CO), 1.60–1.59 (bs, 4H, CH_2), 1.27 (bs, 54H, CH_2), 0.89 (t, J = 7 Hz, 6H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 173.0 (Cq), 170.5 (Cq), 171.2 (Cq), 168.7 (Cq), 169.8 (Cq), 164.4 (Cq), 96.5 (C-1), 73.4 (C-4 o C-5), 70.4 (C-1‴ o C-2″), 68.8 (C-4 o C-5), 65.7 (C-3), 64.6 (C-2″ o C-1‴), 62.4 (C-3‴), 61.3 (C-6), 51.82 (C-2′), 40.8 (C-1″), 39.1 (C-2), 34.3 (*C*H2), 34.0, 31.7 (C-3′), 30.6, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 28.9, 24.9, 24.8, 22.5, 13.6, 0.6; ESI-MS: *m/z* Calcd for $C_{53}H_{94}N_2O_{15}PS$: 1061.61 $[M-H]^-$; found 1061.33.

Synthesis of **6**

Compounds **10** [44] (5.0 mg, 2.8 μmol), **7** (9.1 mg, 20.4 μmol) and PyBOP (17.3 mg, 33 μmol) were dissolved in 2 mL of DMF, and the pH was adjusted to 8–9 by adding DIPEA (19 μL, 56 μmol). The solution was stirred 2 h at room temperature, then the solvent was evaporated under reduced pressure and diethyl ether was added to precipitate the resulting acetylated glycopeptide that was used without further purification. Analytical RP-HPLC: R_t = 14.3 min (C_{18}, 214 nm, 5–40% B in 20 min); ESI^+-MS: *m/z* calcd for $C_{158}H_{206}N_{22}O_{70}S_6$: 3726.2 $[M + H]^+$; found: 3725.7. The crude mixture was dissolved in 5 mL of methanol and NaOMe (pH 8) was added and the solution was stirred at room temperature overnight. The reaction mixture was next neutralized with Dowex 50 W–X8 (H^+) resin, filtered and purified by semi-preparative RP-HPLC to afford **6** after lyophilisation. Yield: 51% (4.3 mg); analytical RP-HPLC: R_t = 7.6 min (C_{18}, 214 nm, 5–40% B in 20 min); ESI^+-MS: *m/z* calcd for $C_{122}H_{170}N_{22}O_{52}S_6Na$: 2990.9 $[M + Na]^+$; found: 2990.2.

Synthesis of **11**

The aldehyde-containing cyclopeptide **13** [45] (2.2 mg, 1.6 μmol) and **α-ONH$_2$-GalNAc** [46–48] (4.4 mg, 0.19 mmol) were dissolved in 0.1% TFA in water (10 mM). After 2 h stirring at 37 °C, the crude mixture was purified by preparative HPLC without additional treatment. Yield: 76% (3.2 mg); analytical RP-HPLC: R_t = 7.7 min (C_{18}, 214 nm, 5–40% B in 20 min); ESI^+-MS: *m/z* calcd for $C_{110}H_{175}N_{28}O_{52}Na$: 2744.20 $[M + Na]^+$; found: 2743.4.

Synthesis of **12**

Compound **12** has been prepared from **13** and hydroxylamine hydrochloride following the procedure described for **11**. Yield: 60% (2.4 mg); analytical RP-HPLC: R_t = 8.6 min (C_{18}, 214 nm, 5–40% B in 30 min); ESI^+-MS: *m/z* calcd for $C_{62}H_{98}N_{22}O_{22}$: 1502.7 $[M + H]^+$; found: 1503.8.

Cell cultures

Peripheral blood mononuclear cells (PBMC) were separated from venous blood of healthy volunteers after their informed consent by density gradient centrifugation using Lymphocite Separation Medium (LSM 1077) (GE Healtcare, Milan, Italy). After cell washing with Phosphate Buffer Saline (PBS) containing 0.1% Fetal Bovine Serum (FBS) (Carlo Erba, Milan, Italy) NK cells were isolated by magnetic separation technique using a negative selection isolation kit (Miltenyi Biotec, Bologna, Italy). Cells were then labelled with a cocktail of biotin-conjugated antibodies against lineage-specific antigens (CD3, CD4, CD14, CD15, CD19, CD36, CD123, CD235a) and labeled with a cocktail of MicroBeads (Miltenyi Biotec). Non-NK cells were separated using a Miltenyi Biotec MACS Column.

NK cells were routinely purified to >90% by this method.

K562 cells are a highly undifferentiated human erythroleukemic cell line, which does not express MHC class I molecules [49], and are a generous gift by Prof. M.C. Mingari (Department of Experimental Medicine, IRCCS AOU San Martino-IST, Genova, Italy).

Human CD1d transfected THP-1 and mouse FF13 iNKT hybridoma cells were kindly provided by Prof. Gennaro De Libero (Department of Research, Experimental Immunology, University Hospital Basel, Basel, CH). All cells were regularly cultured in RPMI-1640 complete growth medium, containing 10% heat inactivated FBS, 100 μg/ml kanamycin (SigmaAldrich, Milan, Italy), 1 mM sodium pyruvate (Lonza, Basel, Switzerland), 2 mM L-glutamime, 1% MEM amino acid solution (Lonza), 0.01 mM β-mercaptoethanol (GE healtcare), and cultured in a humidified atmosphere (5% CO_2, 37 °C).

Calcein AM assay

PBMC cells were labeled with 1 μM Calcein-AM (CAM) (Life Technologies, Monza, Italy) in serum-free PBS for 15 min at 37 °C in the dark. After washing, labelled cells were seeded in 24-well plates and then treated with increasing concentrations (0.1–100 μM) of each compound for 24–48 h at 37 °C in a humidified incubator. After incubation, the cells of each well were harvested, washed, labelled with propidium iodide (PI) (SigmaAldrich), and cell viability was measured by flow cytometry (FACSDiva Option, Becton Dickinson, Milan, Italy). Live cells were identified as CAM^{high}/PI^-, whereas dead cells were CAM^{low}/PI^+. The viability was calculated by FACSDiva software and expressed as the percentage of CAM^{high}/PI^- cell population relative to compound-untreated cells (controls).

NK cell degranulation assay

NK cell degranulation was measured in vitro by determining the expression of CD107a, the lysosome-associated membrane protein-1 (LAMP-1), as previously described [49]. NK cells were untreated/treated with increasing concentrations (0.1–10 μM) of each compound for 24 h in presence of IL-2 (100 U/mL) (Peprotech, Rocky Hill, New Jersey, United States), a cytokine commonly used for NK cell activation [50]. Thereafter, NK cells were incubated with K562 cells at an effector/tumor cell ratio of 1:1 for 1 h at 37 °C in presence of anti-CD107a-Phycoerythrin (PE) (Miltenyi Biotec). Monensin (BD GolgiStop™ reagent, BD Bioscences, Milan, Italy) was added and cells incubated for 3 h at 37 °C. Cells were washed and CD107a expression measured as Mean Fluorescence Intensity (MFI) by using FACSDiva software (BD Bioscience, Milan, Italy). To detect spontaneous NK cell degranulation, a negative control sample (NK cells without K562 target cells) was always included, while to measure the basal response of cells isolated NK cells were stimulated only with IL-2 (100 U/mL) (controls). As positive control we treated cells with 10 μM of 2,3-butanediol, which is a NK cell-activator at this concentration [51, 52].

iNKT cell activation

THP-1 cells (5×10^4 /well) were incubated with increasing concentrations (0.01–100 μM) of each compound or (0.01–100 nM) of α-GalCer, in serum-free medium for 2 h at 37 °C. iNKT hybridoma cells (1×10^5/well) were then added, and the plates incubated in a 37 °C, 5% CO_2, humidified incubator for 48 h. Cell-free supernatants were collected and released IL-2 was measured by standard ELISA assay (R&D systems, Minneapolis, MN) using rat anti-mouse IL-2 mAbs and expressed in pg/ml (mean ± s.d.).

Statistical analysis

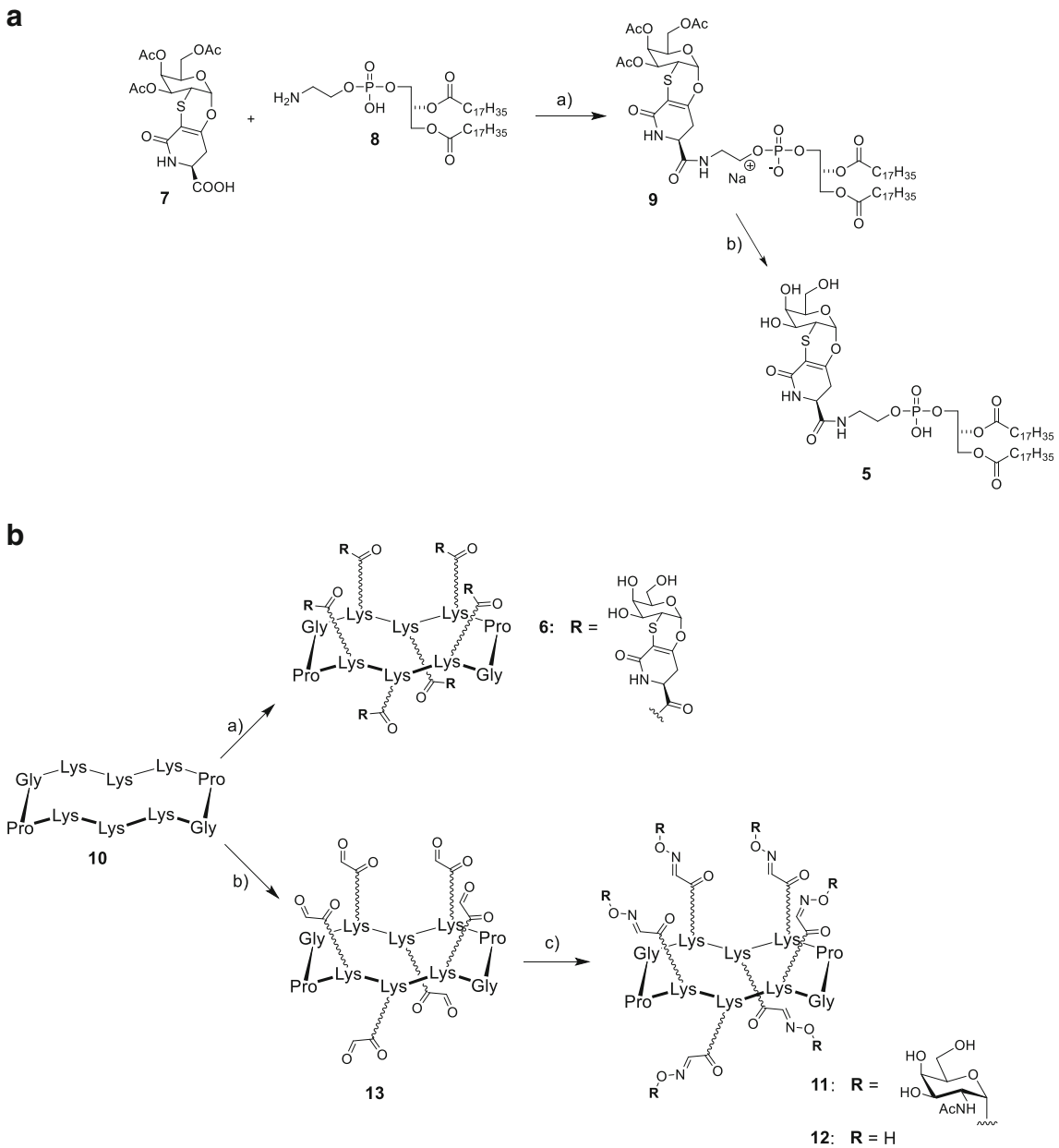
Results were expressed as means ± SEM of at least three independent experiments run in triplicate. Statistical significance was evaluated by the one-way ANOVA followed by Student's *t*-test for unpaired populations (Graph Pad Software, Inc., San Diego, USA). Differences were considered statistically significant when $p \leq 0.05$.

Results and discussion

Chemistry

The glycolipid **5** was prepared starting from the peracetylated derivative **7** [37] (Scheme 1a). The tricyclic scaffold of **7** was efficiently obtained relying on an unconventional and totally stereoselective inverse electron-demand [4 + 2] Diels-Alder reaction between suitably protected glycals and α,α'-dioxothiones as previously described by some of us [37].

The peracetylated derivative **7** (Scheme 1A) was coupled with the commercially available 1,2-distearoyl-sn-glycero-3-phosphoethanolamine **8** using TBTU [*N*,*N*,*N*_,*N*_- tetramethyl-*O*-(benzotriazol-1 yl)uraniumtetrafluoroborate; 2.0 equiv.] and *N*-methyl morpholine (NMM; 2.0 equiv.) as coupling agents. The resulting fully protected glycolipid **9** was obtained in 60% yield as sodium salt, after treatment with a



Scheme 1 **a** Synthesis of the glycolipid **5**; reagents and conditions: a) TBTU, NMM, DMF/$CHCl_3$/CH_2Cl_2, 15 h, rt., then Amberlite IR 120 Na^+, 60%; b) K_2CO_3. MeOH/CH_2Cl_2, 1 h, rt., 45%. **b** Synthesis of the glycoconjugates **11** and **12**; reagents and conditions: a) i: **7**, PyBOP, DIPEA, DMF, rt., 2 h, then ii: MeONa/MeOH, rt., 18 h, 51%; b) i: BocSer(tBu)OH, DIPEA, DMF, rt., 1 h; ii: 0.1% TFA in H_2O, rt., 2 h; iii: $NaIO_4$, water, rt., 30 min, 88% (over 3 steps); c) 0.1% TFA in water, rt., 1 h; α-ONH_2-GalNAc for **11**, 76%; $HONH_2$.HCl for **12**, 60%

cation-exchange resin (Amberlist IR120, Na^+ form). The α-galactosyl **9** was deprotected with a diluted solution of K_2CO_3 to give **5** in 45% yield, after purification by column chromatography on Sephadex LH-20.

Glycopeptide **6** has been prepared from the cyclopeptide scaffold **10** [44] displaying six free lysines and the protected derivative **7** (Scheme 1B). The coupling reaction was performed with PyBOP as coupling reagent in DMF to afford the hexavalent acetylated compound that was subsequently deprotected with sodium methanoate in methanol. The resulting glycopeptide **6** was obtained in 51% yield after RP-HPLC purification Compounds **11** and **12** have been synthesized from cyclopeptide **13** [44] and α-ONH_2-GalNAc [45–48, 53] and hydroxylamine hydrochloride, respectively, using the oxime ligation strategy described earlier [46–48, 53]. The conjugation reaction occurred in water with 0.1% of TFA and the conversion of **13** was found quantitative in both cases within 2 h at 37 °C. After RP-HPLC purification, compounds **11** and **12** were obtained in 76% and 60% yield, respectively.

Biological assays

Cell viability

To evaluate the effect of compounds **1, 5, 6, 8, 11, 12** and **α-OMe-GalNAc** on cell viability human PBMC, containing a variable percentage of NK (from 2% to 18%), and iNKT cells (from 0.1 to 2%) were used [54, 55]. PBMC were labelled with CAM and incubated with increasing concentrations (0.1–100 μM) of each compound for 24/48 h. Cells not exposed to compounds were taken as negative controls. Cell viability, evaluated by FACS, always resulted ≥90% at all concentrations and times tested (see Fig. S1, S2), suggesting that all compounds under study are non-toxic for PBMC and useful for functional studies.

iNKT cell activation

To evaluate the ability of the glycolipid **5** to activate iNKT cells a T cell-antigen presenting assay was used. Mouse iNKT hybridoma cells (FF13; 5×10^4 cells/well), selected for their ability to respond to alpha-galactosylceramide (α-GalCer) presented by CD1d expressing cells [56], were co-cultured with CD1d-transfected THP-1 cells (2.5×10^4 cells/well), previously (2 h) treated with increasing concentrations (0.01–100 μM) of glycolipid **5**. Furthermore, the activity of this compound was compared with the 1,2-distearoyl-sn-glycero-3-phosphoethanolamine **8** and the α-OMe-GalNAc (Fig. 3a), while the α-GalCer (0.01–100 nM) was used as positive control for validation of the assay (Fig. 3b).

IL-2 production, typically measured to assess the abilities of α-GalCer to stimulate human or murine iNKT cell hybridomas [57], was evaluated in the cell culture media after 48 h of co-culture by ELISA.

Figure 3a shows that, as expected, only compounds having a lipid moiety (both the 1,2-distearoyl-sn-glycero-3-phosphoethanolamine **8** and the glycolipid **5**) induced IL-2 release in a concentration-dependent manner.

The maximum effects were: 457 ± 47 and 634 ± 23 pg/mL at 10 μM, and the EC_{50} measured were 0.5 ± 0.02 and 0.7 ± 0.01, respectively. The ability of phospholipid **8** to induce iNKT cell activation has been previously reported by Wu *et al*., [58] and present data are consistent with this report. Conversely, both the α-OMe-GalNAc and the Tn-mimetic **1** failed in inducing iNKT cell activation at all concentrations tested.

Of note, when the Tn-mimetic antigen **1** was conjugated to the phospholipid **8** a significant increase (+ 40 ± 5%; $P \leq 0.01$) in the IL-2 release was measured, compared to the phospholipid **8**.

The overall data clearly demonstrate that the conjugation of Tn-mimetic **1** to a lipid architecture confers to the antigen the ability to activate murine iNKT cells.

NK cell degranulation

To determine the effect of the glycopeptide **6** on NK cell activity a short-term degranulation assay was performed, by measuring the expression of CD107a [51, 52], a cell surface antigen whose expression well correlates with the NK killing activity [49]. The activity of the glycopeptide **6** was compared with that of the α-OMe-GalNAc, the Tn-mimetic **1**, the RAFT-based glycopetide **11** containing six residue of GalNAc, and the hexavalent RAFT-based peptide scaffold **12** (-negative control). Cells treated with 10 μM of 2,3-butanediol, a well-known NK cell-activator [50], were always taken as positive controls (black bar).

As shown in the Fig. 4, both the α-OMe-GalNAc and the Tn mimetic **1** resulted effectively in inducing a higher CD107a expression compared to compound-untreated cells (negative controls, vertical black line). The maximum effects were: +65 ± 13% ($P \leq 0.01$) and +74 ± 26% ($P \leq 0.05$) at 1 μM, respectively.

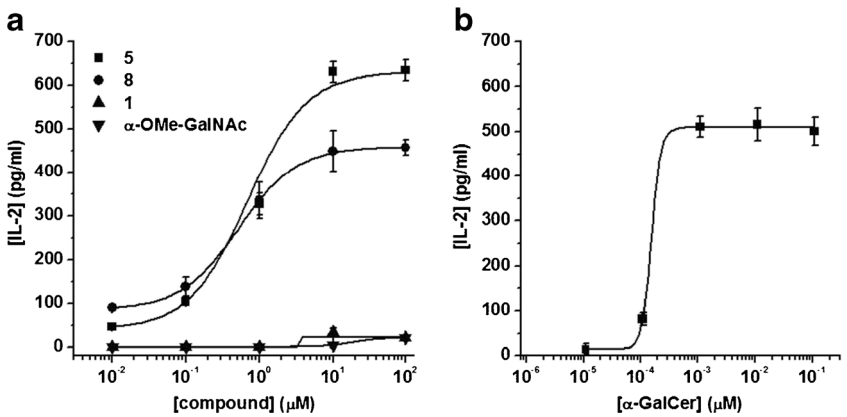


Fig. 3 **a** IL-2 release by iNKT hybridoma cells upon stimulation with **1**, **5**, **8** and α-OMe-GalNAc (0.01–100 μM) and (**b**) α-GalCer (0.01–100 nM)

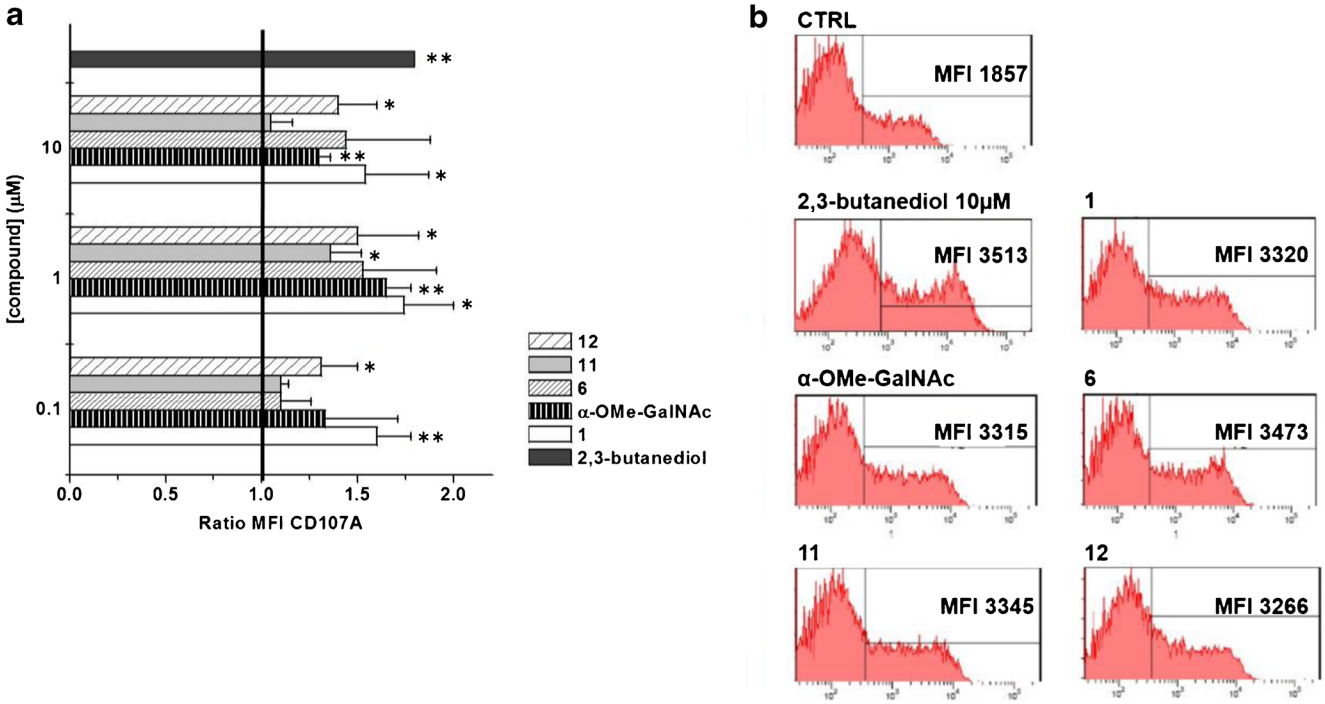


Fig. 4 Expression of CD107A on human NK cells. **a** the increase in CD107A expression on human NK cells after treatment with **1**, **6**, **11**, **12** (0.1–10 μM). Data are expressed as the Mean Fluorescence Intensity (MFI) ratio (treated/control cells). The *black line* represents the expression of CD107A on compound-untreated NK cells (controls). **b** representative examples of CD107A expression (MFI) on NK cells after 1 μM compound treatment. 10 μM 2,3-butanediol was taken as positive control

The conjugation of GalNAc or the Tn mimetic to RAFT peptide (compounds **6** and **11**) did not improve cell responses in comparison to cells treated with monovalent un-conjugated antigens, probably because this type of scaffold is not able to increase the selectivity and the strength of receptor binding, a fundamental feature for eliciting efficient immune cell activations. Conversely, the RAFT-based peptide **12** resulted effective at all the concentrations tested.

The overall results suggest that a peptide RAFT is an unsuitable scaffold for the multivalent presentation of these type of molecules, since itself could interact with receptors, expressed on NK cells, specifically recognizing peptides.

From our data it is, however, reasonable to conclude that both α-OMe-GalNAc and Tn mimetic **1** are able to activate human NK cells, as 2,3-butanediol (positive control) does.

Conclusion

In summary, we reported here the synthesis of the glycolipid **5** and the hexavalent glycopeptide **6,** containing one and six copies of the rigid α-Tn mimetic **1**, respectively. From our data: i) all the tested compounds are biocompatible; ii) the Tn mimetic **1** activates human NK cells and, when suitably conjugated to a glycolipid, murine iNKT cells as well.

Since to induce a strong and efficient tumour immunesurveillance a complex cross-talk between the innate and adaptive immune system is strictly needed, the compound **1,** able to activate both iNKT and NK cells, appears to be an appealing lead, useful to act as a linker between the two immune systems for promoting efficient immune responses.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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